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First Named Inventor:	Mark Bobrow
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TRANSMITTAL

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Title of Invention	2-difluoromethylphenyl-containing substrates for an analyte dependent enzyme activation system

Application Number:
Date:
First Named Applicant: Dr. Mark Norman Bobrow

Confirmation Number:
Attorney Docket Number:

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Documents being submitted	Files
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	us-request.dtd
	us-request.xsl
us-fee-sheet	CARDP-usfees.xml
	us-fee-sheet.xsl
	us-fee-sheet.dtd
application-body	CARDAP1-trans.xml

	us-application-body.xsl
	application-body.dtd
	wipo.ent
	mathml2.dtd
	mathml2-qname-1.mod
	isoamsa.ent
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FEE TRANSMITTAL

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Title of Invention	2-difluoromethylphenyl-containing substrates for an analyte dependent enzyme activation system

Application Number:
Date:
First Named Applicant: Dr. Mark Norman Bobrow
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Title of Invention	2-difluoromethylphenyl-containing substrates for an analyte dependent enzyme activation system

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Electronic Version

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Description

2-difluoromethylphenyl-containing substrates for an analyte dependent enzyme activation system

DETAILED DESCRIPTION

[0001] Catalyzed reporter deposition (CARD) is a novel method of signal amplification which constitutes the subject matter of U.S. Pat. Nos. 5,731,158, 5,583,001 and 5,196,306. It is also discussed, and assays described in Bobrow et al., Journal of Immunological Methods, 125: 279-285 (1989) and in Bobrow et al., Journal of Immunological Methods, 137:103-112 (1991).

[0002] The CARD method utilizes an analyte-dependent enzyme activation system ("ADEAS") to catalyze the deposition of reporter or hapten groups (labels) onto a solid phase or proteins. These enzymatically deposited labels are detected directly or indirectly, resulting in signal amplification and improved detection limits. In the previously disclosed references, a peroxidase was the preferred enzyme.

[0003] The present invention involves methods and compounds for use with hydrolytic enzymes such as esterases, alkaline phosphatase and beta-galactosidase. These methods offer advantages over peroxidase based methods in that they are slower and more linear, allowing for greater

control and dynamic range.

[0004] As disclosed herein, the present invention relates to the use of 2-difluoromethylphenyl-containing compounds incorporated into hydrolase substrates. The term analyte dependent enzyme activation system (ADEAS) refers to an enzyme system wherein (i) at least one enzyme is coupled, in any manner known to those skilled in the art, to a member of a specific binding pair, or (ii) the enzyme need not be coupled to a member of a specific binding pair when it is the analyte. The enzyme, either by itself or in connection with a second enzyme, catalyzes the formation of a reactive intermediate which then is deposited wherever there is a receptor for the reactive intermediate.

[0005] The term surface as used herein means any solid support or phase known to those skilled in the art including, but not limited to cells, tissues, membranes, slides, beads and the surface of proteins.

[0006] The term amplification as used herein means amplification of reporter signal.

[0007] The term reactive intermediate means the 2-difluoromethylphenyl-containing compounds have been primed by the enzyme to bind to the receptor.

[0008] The term receptor means a site which will bind to the reactive intermediate through the formation of a covalent bond.

[0009]

The term detectably labeled means that the 2-difluoromethylphenyl-

containing compounds, in addition to the substrate characteristics, are coupled to a reporter or an unlabeled first member of a specific binding pair. In the case in which the compound is coupled to an unlabeled member of a specific binding pair, after the reactive intermediate is covalently bound to the receptor, the substrate-specific binding pair complex is reacted with the second member of the binding pair which is coupled to a reporter.

[0010] Members of specific binding pairs suitable for use in practicing the invention can be of the immune or non-immune type. Immune specific binding pairs are exemplified by antigen/antibody systems or hapten/anti-hapten systems such as dinitrophenyl (DNP)-anti-DNP. The antibody member, whether polyclonal, monoclonal or an immunoreactive fragment thereof, of the binding pair can be produced by customary methods familiar to those skilled in the art. The terms immunoreactive antibody fragment or immunoreactive fragment mean fragments which contain the binding region of the antibody. Such fragments may be Fab type fragments which are defined as fragments devoid of the Fc portion, e.g., Fab, Fab' and F(ab')2 fragments, or may be so-called "half molecule" fragments obtained by reductive cleavage of the disulfide bonds connecting the heavy chain components of the intact antibody. If the antigen member of the specific binding pair is not immunogenic, e.g., a hapten, it can be covalently coupled to a carrier protein to render it immunogenic.

[0011] Non-immune binding pairs include systems wherein the two

components share a natural affinity for each other but are not antibodies. Exemplary non-immune binding pairs are biotin-avidin or biotin-streptavidin, folic acid-folate binding protein, complementary probe nucleic acids, etc. Also included are non-immune binding pairs which form a covalent bond with each other. Exemplary covalent binding pairs include sulfhydryl reactive groups such as maleimides haloacetyl derivatives and amine reactive groups such as isothiocyanates, succinimidyl esters, sulfonyl halides, and coupler dyes such as 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethyl-amino) benzoic acid (DMAB), etc.

[0012] The term deposition means directed binding of a reactive intermediate to the receptor which results from the formation of a covalent bond.

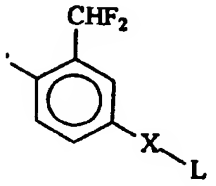
[0013] The enzyme catalyzes the deposition of a 2-difluoromethylphenyl-containing compound by converting the compound to a reactive intermediate which is capable of covalently binding to a receptor.

[0014] The present invention also concerns 2-difluoromethylphenyl-containing compounds which heretofore have not been described as enzyme substrates.

[0015] Enzymes suitable for use with 2-difluoromethylphenyl-containing compounds of the present invention include hydrolases. More particularly, phosphatases, glycosidases and esterases can be employed. One particularly preferred enzyme which is suitable for the novel substrates of the invention is alakaline phosphatase.

[0016] A wide variety of detectable labels are available for linking to the 2-difluoromethylphenyl-containing moiety, and the present invention is limited to any specific label. The detectable label can be a reporter as a radioactive isotope such as ^{125}I, enzymes, fluorescent reagents or groups such as fluorescein, tetramethylrhodamine, cyanine dyes, Alexa dyes or BODIPY dyes, chemiluminescent reagents or groups, or electrochemical materials. The detectable label can also be a member of a specific binding pair as described above. Other labels will be readily apparent to one of skill in the art.

[0017] Compounds for use in assays of the present invention generally have the structure:



wherein Y is a moiety capable of being cleaved by a hydrolytic enzyme, L is a detectable label and X is a group linking L to the 2-difluoromethylphenyl moiety.

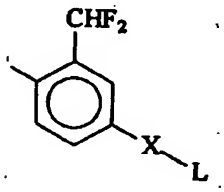
[0018] Moieties capable of being cleaved by hydrolytic enzymes include phosphate esters, glycoside esters such as galactose and glucose, and alkyl esters cleavable by non-specific esterases.

[0019]

The linker, X, group can be virtually any linker group capable of linking the detectable label to the 2-difluoromethylphenyl moiety, and the

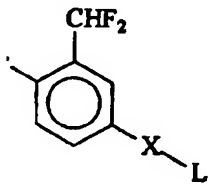
invention is not limited to the use of any specific linkers. Any linear or branched alkyl (C_1 to C_{10} for example) or aryl group can serve as a linker, the only requirement being that it links the 2-difluoromethylphenyl moiety with the label.

[0020] In another embodiment, novel compounds which are substrates for hydrolytic enzymes are disclosed. One example of such a compound is:



wherein Y is a group capable of being cleaved by a hydrolytic enzyme, L is a reporter and X is a group linking L to the 2-difluoromethylphenyl moiety.

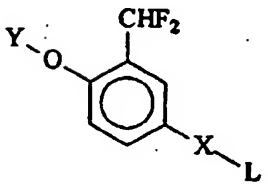
[0021] A second example of a compound which is a substrate for hydrolytic enzymes is:



wherein Y is phosphate, L is a detectable label and X is a group linking L to the 2-difluoromethylphenyl moiety.

Claims

[c1] In an assay for detecting or quantitating an analyte, employing an analyte dependent enzyme activation system which reacts with a substrate portion of a conjugate which comprises a detectably labelled substrate for said enzyme, so as to form an activated conjugate, which activated conjugate covalently binds to a site on a surface having a receptor for said activated conjugate, said receptor not being reactive with the analyte dependent enzyme activation system, wherein the detectably labeled portion of the bound conjugate either directly or indirectly generates a signal which is detected or quantitated, the improvement comprising: using as said conjugate a 2-difluoromethylphenyl-containing compound having the structure:



wherein Y is a moiety capable of being cleaved by a hydrolytic enzyme, L is a detectable label and X is a group linking L to the 2-difluoromethylphenyl moiety.

[c2] The assay according to claim 1, wherein Y is phosphate and L is a first member of a specific binding pair.

[c3] The assay according to claim 2, wherein L is biotin or dinitrophenyl

[c4] The assay according to claim 1, wherein Y is phosphate and L is a fluorescent group.

[c5] The assay according to claim 4, wherein L is fluorescein, tetramethylrhodamine, sulforhodamine 101, a cyanine dye, Alexa dye or BODIPY dye.

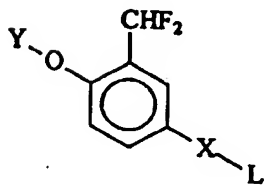
[c6] The assay according to claim 1, wherein Y is a glycoside and L is a first member of a specific binding pair.

[c7] The assay according to claim 6, wherein L is biotin or dinitrophenyl

[c8] The assay according to claim 1, wherein Y is a glycoside and L is a fluorescent group.

[c9] The assay according to claim 8, wherein L is fluorescein, tetramethylrhodamine, sulforhodamine 101 a cyanine dye, Alexa dye or BODIPY dye.

[c10] A 2-difluoromethylphenyl-containing compound having the structure:



wherein Y is a group capable of being cleaved by a hydrolytic enzyme, L is a reporter and X is a group linking L to the 2-difluoromethylphenyl moiety.

[c11] A compound according to claim 10, wherein L is a fluorescent group.

[c12] A compound according to claim 11, wherein L is fluorescein, tetramethylrhodamine, sulforhodamine 101, a cyanine dye, Alexa dye or BODIPY dye.

[c13] A compound according to claim 10 wherein Y is phosphate.

[c14] A compound according to claim 10 wherein Y is phosphate and L is a fluorescent group.

[c15] A compound according to claim 14, wherein L is fluorescein, tetramethylrhodamine, sulforhodamine 101, a cyanine dye, Alexa dye or BODIPY dye.

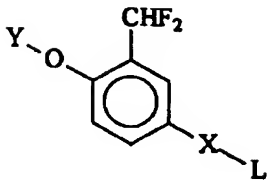
[c16] A compound according to claim 10 wherein Y is a glycoside.

[c17] A compound according to claim 10 wherein Y is a glycoside and L is a fluorescent group.

[c18] A compound according to claim 17 wherein L is fluorescein, tetramethylrhodamine, sulforhodamine 101, a cyanine dye, Alexa dye or BODIPY dye.

[c19] A 2-difluoromethylphenyl-containing compound having the

structure:

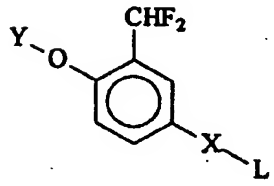


wherein Y is phosphate, L is a first member of a specific binding pair and X is a group linking L to the 2-difluoromethylphenyl moiety.

[c20] A compound according to claim 19, wherein L is biotin.

[c21] A compound according to claim 19, wherein L is dinitrophenyl.

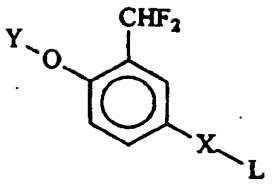
[c22] A 2-difluoromethylphenyl-containing compound having the structure:



wherein Y is a glycoside, L is a first member of a specific binding pair other than biotin and X is a group linking L to the 2-difluoromethylphenyl moiety.

[c23] A compound according to claim 22, wherein L is dinitrophenyl.

[c24] A 2-difluoromethylphenyl-containing compound having the structure:



wherein Y is a glycoside other than galactose, L is a first member of a specific binding pair, and X is a group linking L to the 2-difluoromethylphenyl moiety.

2-difluoromethylphenyl-containing substrates for an analyte dependent enzyme activation system

Abstract

This invention relates to 2-difluoromethylphenyl-containing compounds and the use of 2-difluoromethylphenyl-containing compounds as hydrolytic enzyme substrates in a variety of applications such as catalyzed reporter deposition